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Award Number: W81XWH-04-1-0200

TITLE: Vasculature-Specific Adenovirus Vectors for Gene Therapy of Prostate Cancer

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REPORT DATE: February 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-02-2006	Annual	1 Feb 2005 - 31 Jan 2006

4. TITLE AND SUBTITLE
Vasculature-Specific Adenovirus Vectors for Gene Therapy of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0200

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas MD Anderson Cancer Center
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

In Year 2 of the project we have completed Task 2 (as per approved Statement of Work) by characterizing the binding specificity of the Ad fiber proteins designed to bind IL-11Rα. In Task 3 of the project we identified the most promising fiber-ligand constructs and designed Ad genomes that incorporate the genes for these proteins in addition to the reporter-expressing gene cassettes.

15. SUBJECT TERMS
Gene Therapy, Adenovirus Vector, Genetic Engineering, Receptor-Specific Targeting, Angiogenesis, Vasculature Endothelium, Endothelium-Specific Expression, Light-Based Imaging.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:

The scope of the proposed work is to develop novel Ad vectors, which will be targeted to vasculature of prostate tumors via genetic incorporation into their capsid of the recently identified endothelium-specific peptides. Additionally, tumor selectivity of these vectors is to be further improved by limiting the expression of the therapeutic transgene carried by the vectors, to endothelial cells of tumor vasculature. These double-targeted viruses are to be able to selectively infect blood vessels within the tumors and destroy the blood supply to tumors by locally expressing "suicide" transgene.

REPORT BODY:

In our previous progress report (Year 1) we presented the work accomplished in Task 1 of the project. Therefore, the work in the Year 2 of the project started with the experiments described in Task 2. This report covers a twelve-month period of work (months 9-21, as outlined in the originally proposed timeline).

Description of work:

Task 2. Assessment of the receptor-binding capability of the modified fiber proteins.

As per our Statement of Work, this Task was to include the ELISA-based analyses of the capacity of the proteins produced in Task 1 to bind to recombinant form of cognate cellular receptor, IL-11Rα.

Experiments and results:

The evaluation of the interleukin-11 receptor α (IL-11Rα)-binding by the newly derived fiber proteins by ELISA was done as follows. The pVS-derived expression vectors that encoded the fiber proteins modified with the IL-11Rα–specific peptides (*GRRAGGS, CGRRAGGSC, or VGRRAGGSA*) were used to transfect 293T cells to direct the fibers' expression. The expression of the fibers was then confirmed by Western Blot of the lysates of the transfected cells. These lysates were then used in ELISA with purified IL-11Rα protein (Santa Cruz Biotechnologies, Santa Cruz, CA). In parallel, purified recombinant extracellular fragment of the Ad5 receptor CAR, sCAR, was used as a control for binding of the transiently expressed wild type Ad fiber protein to confirm that our ELISA technique works. Also included in this experiment was the recombinant phage containing the *CGRRAGGSC* peptide (1), which was provided to us by Dr. Renata Pasqualini (University of Texas M.D. Anderson Cancer Center).

This assay showed excellent binding of the wt fiber to sCAR and no binding of the peptide-modified fibers to IL-11Rα. Most surprisingly, however, was the lack of binding to IL-11Rα by the phage vector (Table 1).

Table 1. ELISA with the lysates of 293T cells transiently expressing fiber-peptide proteins. All fiber constructs shown contained the targeting peptide at the carboxy terminus of the double-mutated Ad5 fiber (FΔ2) that is unable to bind to sCAR.

Fiber protein present in the lysate	OD_{490}	
	sCAR	IL-11R
none (negative control)	0.0703	0.1222
wt Ad5 fiber (positive control for sCAR binding)	2.2582	0.1214
FΔ2, (negative control for sCAR binding)	0.0733	0.1266
FΔ2.Fc-*GRRAGGS*	0.0693	0.1191
FΔ2.Fc-*CGRRAGGSC*	0.0725	0.1265
FΔ2.Fc-*VGRRAGGSA*	0.0725	0.1544
fd-IL11R, phage displaying the peptide	0.2178	0.3310
fd-tet, phage with unmodified pIII (negative control)	0.0603	0.1342
anti-IL11R Ab N-20 (Santa Cruz Biotech.)	0.0354	0.0764

In this regard, it should be mentioned that the overall quality of the reagents, which we purchased from Santa Cruz Biotechnologies, was rather poor: none of the several anti-IL-11Rα antibodies (Ab) worked with the lysate of the IL-11Rα-positive LNCaP cells (8) in Western blot. In addition, these Ab did not detect purified IL-11Rα protein in ELISA, and detected it in Western blot only when the protein was used in the amount of 1μg. Of note, alternative commercial sources of anti-IL-11Rα Ab and IL-11Rα could not be found. Therefore, we hypothesized that the lack of binding to purified IL-11Rα in ELISA, which we had seen with both the cell lysates and the phage, could be due to low quality of this protein.

To troubleshoot these unexpected findings, two additional efforts were undertaken. First, we chose to test our recombinant fiber constructs in the most stringent cellular system, in which two isogenic cell lines that would differ only by the level of the IL-11Rα expression would serve as targets for these fiber proteins. To this end, we made an IL-11Rα–expressing derivative of the IL-11Rα–negative 293 cell line and use it in combination with the parental 293 cells to test our constructs. IL-11Rα cDNA (Open Biosystems, Huntsville, AL) was linked *via* an internal ribosome entry sequence (IRES) to the neomycin aminotransferase gene II (G418 resistance) within the mammalian expression plasmid pIRES-neo3 (Clontech, Mountain View, CA). The

resultant plasmid, pIRES.IL11R, was used to transfect 293 cells and the G418-resistant cells were selected. Expression of IL-11Rα in these 293-IL11R cells was demonstrated by quantitative RT-PCR. In addition, we have designed a recombinant protein comprising the Ad5 fiber-T4 fibritin chimera (used here as a scaffold) fused at its carboxy terminus with the human IL-11; expressed it transiently in 293T cells; and used the lysate of these cells as a probe in a flow cytometry experiment with 293-IL11Rα cells, which confirmed the presence of the receptor on these cells.

Next, these 293-IL11Rα cells were used for FACS analysis to evaluate the phage containing the *CGRRAGGSC* peptide. Unfortunately, the data obtained in this experiment was no different from the ELISA results presented above: the phage showed no preferential binding to 293-IL11Rα cells (as compared to parental 293 cells) (Table 2).

Table 2. Flow cytometry analysis of phage binding to cell-associated target receptor. Binding to IL-11Rα-expressing cells of the GRRAGGS peptide-modified phage (fd-GRRAGGS) was compared to that of the unmodified parental phage, fd-tet. Another phage vector, fuCT/6C6, that contains a single chain Ab to PSMA (prostate specific membrane antigen) and the PSMA-expressing derivative of 293 cells 293/PSMA were used as positive control of binding.

Cells	Phage	Mean fluorescence
293	fd-tet	11.13
293	fd-GRRAGGS	11.15
293	fuCT/6C6	9.47
293/IL11Rα	fd-tet	7.99
293/IL11Rα	fd-GRRAGGS	8.3
293/IL11Rα	fuCT/6C6	12.64
293/PSMA	fd-tet	6.07
293/PSMA	fd-GRRAGGS	11.51
293/PSMA	fuCT/6C6	181.41

The availability of 293-IL11Rα cells also made it possible to test the IL-11Rα binding of the newly made fiber proteins in the context of a modified Ad particle. Such additional experiments would provide us with a clear answer to whether the incorporation of these modified fibers into an Ad virion makes the virus IL-11Rα-specific. The viruses containing the peptide-modified fibers were generated by using a fiber trans-complementation method described by VonSeggern *et al.* (6). Briefly, a “tester” Ad vector, Ad5LucF^0, that expresses a firefly luciferase

and also lacks the fiber gene in its genome, was amplified in 293/F28 cells that constitutively express wild type Ad5 fiber (5). Next, the pVS-derived plasmids expressing the peptide-modified fibers (above) were used to transfect 293T cells, which were then infected with the Ad5LucF^0. This resulted in simultaneous replication of the fiber-less virus and the expression of the candidate fibers in each 293T cell that has been both transfected and infected. As a result, the viruses generated in this experiment each contained one of the peptide-modified fiber candidates (confirmed by Western blot of the virions purified on CsCl). These viruses were used for parallel infections of 293 and 293-IL11Rα cells. The result of this experiment corroborated the data, which we previously obtained with the *GRRAGGS*-modified fiber proteins in the ELISA and FACS formats (above): no IL-11Rα-specific gene transfer was observed (Fig. 1).

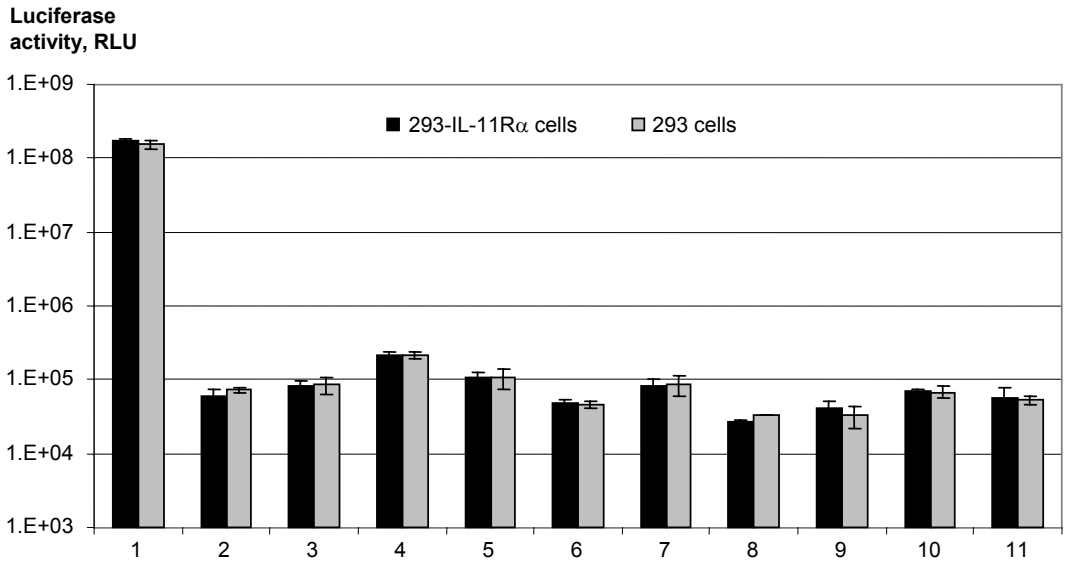


Figure 1. Transduction of 293 and 293/IL-11Rα cells with the fiber-modified Ad vectors. Cells were infected with Ad vectors obtained with the fiber trans-complementation method. Twenty-four hours post infection the cells were collected, lyzed and the activity of the Ad vector-expressed luciferase was measured. Viruses used in this experiment incorporated the fiber proteins with peptides inserted in either the extended HI loop (FΔ2.PB10 or FΔ2.PB40), or the carboxy terminus (FΔ2.Fc). The fiber constructs contained in each of the Ad vector are: 1. wild type Ad5 fiber, 2. mutated FΔ2, 3. FΔ2.PB10-GRRAGGS, 4. FΔ2.PB10-CGRRAGGSC, 5. FΔ2.PB10- VGRRAGGSA, 6. FΔ2.PB40-GRRAGGS, 7. FΔ2.PB40-CGRRAGGSC, 8. FΔ2.PB40- VGRRAGGSA, 9. FΔ2.Fc-GRRAGGS, 10. FΔ2.Fc-CGRRAGGSC, 11. FΔ2.Fc-VGRRAGGSA.

Conclusion: While it is feasible that a receptor-specific peptide has lost its binding capability upon genetic fusion with the Ad fiber protein (such failures have been reported previously (3, 7)), the lack of any binding to IL-11Rα by the *GRRAGGS*-displaying phage particles makes us believe that the identification of IL-11Rα as a target for the *GRRAGGS* peptide (1), which provided the rationale for IL-11Rα targeting in this project, was done incorrectly. This conclusion is further supported by the data from Dr. Chun Li (University of Texas M.D. Anderson Cancer

Center), who has demonstrated that radiolabelled *GRRAGGS* peptide does not bind to IL-11Rα-expressing cells (of note, these studies were done outside of the scope of this project, and have not been supported by this award). Based on this conclusion, we would like to propose that the fiber proteins designed in this project to target IL-11Rα-expressing cells are excluded from the future work, and the work in Year 3 is focused entirely on the fiber constructs that have other targets in the tumor vasculature.

Task 3. Generation and preliminary characterization of Ad vectors incorporating the flt-1 promoter-controlled dual expression cassette and the fibers modified with targeting peptides.

- *Construction within an Ad shuttle vector of two flt-1 promoter-driven expression cassettes incorporating HSV TK and luciferase genes.*
- *Derivation of an Ad rescue vector containing Ad genome with these expression cassettes.*
- *Transfer of the modified fiber genes from mammalian expression vectors into the Ad fiber shuttle vector*.
- *Generation of a series of Ad genomes incorporating both the expression cassettes and the modified fiber genes*.

Experiments and results:

The overall design of the expression cassettes to be incorporated into Ad vectors was slightly modified compared to the originally proposed. Specifically, instead of inserting two similarly designed cassettes, each containing one of the two transgenes, into the Ad rescue vectors, we chose to design one cassette to express both transgene products, the HSV TK and luciferase. This was done to avoid potential intramolecular recombination events involving the two copies of the promoter and polyadenylation sequences, which otherwise would be present in each rescue plasmid. To obviate the necessity for two expression cassettes, we chose to use a genetic fusion of HSV TK and firefly luciferase, "TL" for short, whose gene was kindly provided to us by Dr. Vladimir Ponomarev (Memorial Sloan-Kettering Cancer Center, New York, NY).

First, the open reading frame of the TL gene was cloned into the pShuttle-CMV vector (Stratagene, La Jolla, CA), resulting in pShuttle.CMV.TL, in which the TL sequence was under transcriptional control of cytomegalovirus immediate-early promoter. Next, the promoter-less pShuttle (Stratagene) was used to subloclone an flt-1 promoter that was provided to us by our Consultant Dr. Paul Reynolds (Royal Adelaide Hospital, North Terrace, Australia), and the TL sequence downstream of it, yielding pShuttle.Flt.TL.

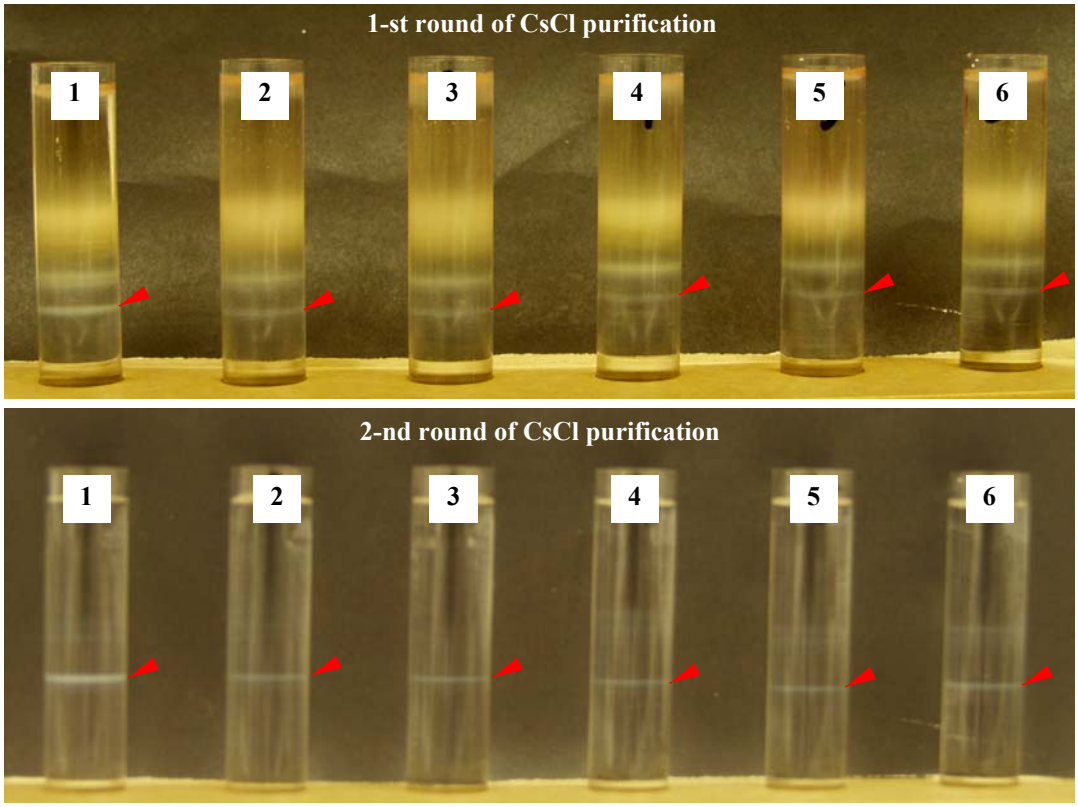
Both expression cassettes were then transferred from these shuttle plasmids into the rescue vector pVK500C (2) to replace the E1 region of Ad5 genome. This was done by homologous DNA recombination in bacteria as previously described (4). The resultant Ad rescue vectors incorporating the CMV promoter- and FLT promoter-containing expression cassettes were designated, pVK514 and pVK515, respectively.

In parallel, we narrowed down the number of the fiber protein candidates, whose genes were considered for subsequent incorporation into Ad genomes. Based on the protein trimerization data generated in Year 1 of the project, the priority was given to those fiber-peptide species that showed satisfactory trimerization profiles. Thus, the following nine combinations of peptides and sites of incorporation within the fiber molecule were identified for further evaluation:

- peptides *SMSIARL*, *CSMSIARLC*, *VSMSIARLA*, *VSFLEYR*, *CAGGVAGGC*, and *VAGGVAGGA* incorporated within the extended HI loop of the mutated fiber (the scaffold designated FΔ2.PB40)
- peptides *SMSIARL*, *VSFLEYR*, and *AGGVAGG* fused to the carboxy terminus of the mutated fiber (the scaffold designated FΔ2.Fc).

To further narrow our choices we tested the efficacy with which each of these modified fibers incorporates into Ad capsid. For this, we used the above mentioned fiber trans-complementation protocol. In brief, expression plasmids encoding each of the nine fiber candidates was used to complement the fiber deficiency of the tester virus, Ad5LucF^0 (above). The resultant viruses were purified on two CsCl gradients (Fig. 2), and analyzed by Western blot with anti-fiber Ab (Fig. 3). Based on the results of these assays, the following fiber-peptide constructs were selected for the virus rescue work in Year 3: FΔ2.PB40-*SMSIARL*, FΔ2.Fc-*VSFLEYR*, and FΔ2.PB40-*VAGGVAGGA*.

The genes corresponding to these protein constructs were transferred to the fiber shuttle vector pZ3.1. These genes were then excised from the resultant plasmids together with the flanking Ad DNA sequences, and used for homologous DNA recombination with the linearized pVK514 and pVK515. The plasmids obtained each contained a complete Ad5 genome with a TL-expressing cassette driven by either the CMV or Flt promoter, and one of the genes encoding for a peptide-modified fiber.



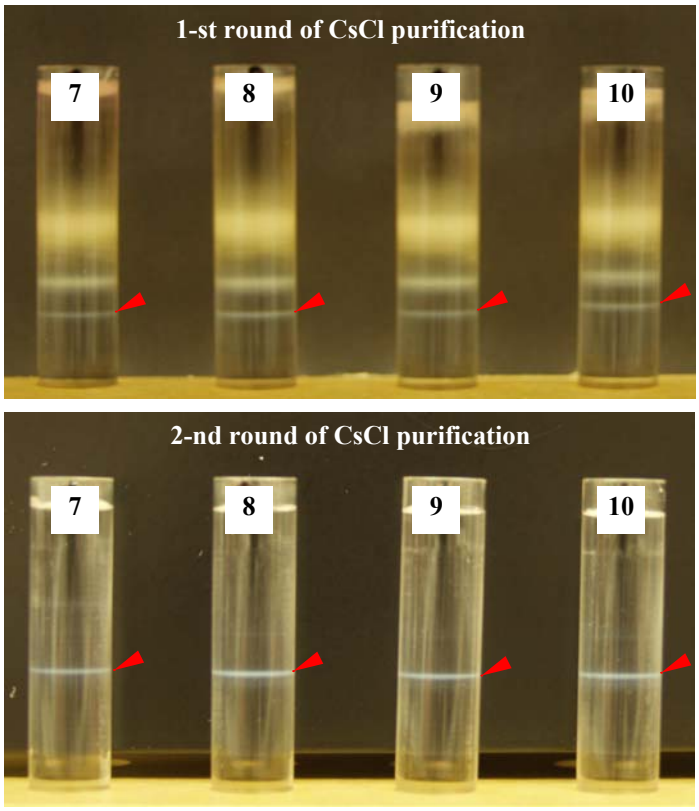


Figure 2. Purification of Ad vectors prepared by fiber trans-complementation method. Bands containing complete Ad virions are shown by red arrowheads. Fiber proteins used in each instance are listed below.

1. wild type Ad5 fiber (control)
2. FΔ2.PB40-*SMSIARL*
3. FΔ2.PB40-*CSMSIARLC*
4. FΔ2.PB40-*VSMSIARLA*
5. FΔ2.PB40-*VSFLEYR*
6. FΔ2.Fc-*SMSIARL*
7. FΔ2.Fc-*VSFLEYR*
8. FΔ2.PB40-*CAGGVAGGC*
9. FΔ2.PB40-*VAGGVAGGA*
10. FΔ2.Fc-*AGGVAGG*

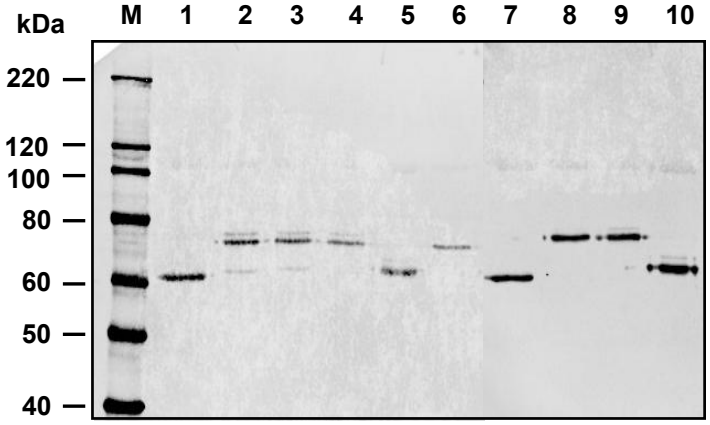


Figure 3. Incorporation of the modified fiber proteins in Ad particles. CsCl-purified Ad vectors produced using the fiber trans-complementation method were fully denatured and analyzed by Western blot with anti-fiber Ab. Fiber proteins tested are listed below. M, 1kDa protein ladder

1. wild type Ad5 fiber (control)
2. FΔ2.PB40-*SMSIARL*
3. FΔ2.PB40-*CSMSIARLC*
4. FΔ2.PB40-*VSMSIARLA*
5. FΔ2.Fc-*SMSIARL*
6. FΔ2.PB40-*VSFLEYR*
7. FΔ2.Fc-*VSFLEYR*
8. FΔ2.PB40-*CAGGVAGGC*
9. FΔ2.PB40-*VAGGVAGGA*
10. FΔ2.Fc-*AGGVAGG*

KEY RESEARCH ACCOMPLISHMENTS:

- The results of our rigorous analysis of binding to IL-11Rα suggested that the published data on the specificity of one of the leading peptide candidates could be incorrect and also suggested elimination of this peptide ligand from future work.
- The most promising fiber-peptide combinations identified in preliminary work in Year 1 of the project were tested in a more stringent assay. These new data allowed us to narrow the selection of the protein construct worth further consideration in this project.
- Recombinant genomes of six Ad vectors containing both the reporter gene cassettes and the modified fiber genes have been constructed.

REPORTABLE OUTCOMES: none at this early point.

CONCLUSIONS:

We have been able to identify those modified fiber constructs that are most likely to function as Ad targeting moieties upon incorporation into Ad virions. Also, we have generated recombinant Ad genomes that incorporate the genes encoding these fiber proteins in addition to the reporter-encoding gene cassettes.

During the reported period the work progressed along the lines of the work plan outlined in the Statement of Work. Our experimental work remains to be behind the schedule by approximately 2-3 months. This delay was caused by our relocation to MD Anderson Cancer Center immediately before this project was funded, and also by the additional fiber-complementation experiments that we chose to do in order to characterize our fiber constructs more fully. To move more quickly in Year 3 of the project we have recently hired a Research Assistant who will help us with the experimental work in Year 3. This position will be fully supported by this PI start-up funds at least through July 2006. No additional funds are required for its support.

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